# Wild type agr-negative livestock-associated MRSA exhibits high adhesive capacity to human and porcine cells

Bünter, Julia P ; Seth-Smith, Helena M B ; Rüegg, Simon R ; Heikinheimo, Annamari ; Borel, Nicole ; Johler, Sophia

Abstract: Methicillin-resistant Staphylococcus aureus (MRSA) is one of the leading causes of nosocomial infections and a major public health concern worldwide. During the last decade, MRSA of CC398 have emerged as important colonizers of livestock. These strains also represent an increasing cause of human infections. A recent study reporting a new dominant spa type among MRSA from Finish fattening pigs (CC398/t2741) identified a strain lacking both the global virulence regulator gene locus agr and the adhesion gene fnbB. The aim of this study was to characterize this agr/fnbB-negative livestock-associated MRSA strain in terms of growth, hemolysis and adhesive capacity, and to provide data on its genomic background. To this end, growth curves and hemolysis patterns were generated and adhesion assays on human keratinocyte and porcine nasal mucosa cell lines were performed. Whole genome sequencing was used to determine the nature and extent of the relevant deletions in the livestock strains. For comparison, an agr-positive, fnbB-negative CC398/t2741 strain from the same pig herd, an agr/fnbB-positive CC398/t034 strain from another pig herd and one human MRSA strain and its isogenic Δagr knockout mutant were used. The agr-negative strains adhered significantly better to human and porcine host cells than the agr-positive control strains. For the agr-positive porcine MRSA strains, cytotoxic effects on porcine mucosal cells were observed. The strong adhesive capacity of the naturally agr-negative livestock-associated MRSA, in combination with diminished cytotoxic effects, is likely favorable for inducing persistent colonization in pigs. Independently of the host cell type, similar adhesive capacities of the naturally agr-negative livestock-associated MRSA and the human MRSA strain were shown. Our results indicate that loss of agr in the livestock-associated MRSA strain investigated in this study may have increased its potential to be transmitted to and amongst humans.

DOI: https://doi.org/10.1016/j.resmic.2016.09.006

# Wild type *agr*-negative livestock-associated MRSA exhibits high adhesive capacity to human and porcine cells

Julia P. Bünter[1], Helena M.B. Seth-Smith[1], Simon Rüegg[2], Annamari Heikinheimo[4], Nicole Borel[1*¶], Sophia Johler[3¶]

[1] Institute for Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 268, CH-8057 Zurich, Switzerland

[2] Section of Epidemiology, Vetsuisse Faculty, University of Zürich, Winterthurerstrasse 270, CH-8057 Zurich, Switzerland

[3] Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 272, CH-8057 Zurich, Switzerland

[4] Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Agnes Sjöberginkatu 2, P.O. Box 66, FI-00014 Helsinki, Finland

* Corresponding author

Email: n.borel@access.uzh.ch (NB)

¶These authors contributed equally to this work.

# Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) are one of the leading causes of nosocomial infections and a major public health concern worldwide. During the last decade, MRSA of CC398 have emerged as important colonizers of livestock. These strains also represent an increasing cause of human infections. A recent study reporting a new dominant *spa* type among MRSA from Finish fattening pigs (CC398/t2741) identified a strain lacking both the global virulence regulator gene locus *agr* and the adhesion gene *fnbB*. The aim of this study was to characterize this *agr/fnbB*-negative livestock-associated MRSA strain in terms of growth, hemolysis, and adhesive capacity, and to provide data on its genomic background. To this end, growth curves and hemolysis patterns were generated and adhesion assays on human keratinocyte and porcine nasal mucosa cell lines were performed. Whole genome sequencing was used to determine the nature and extent of the relevant deletions in the livestock strains. For comparison, an *agr*-positive, *fnbB*-negative CC398/t2741 strain from the same pig herd, an *agr/fnbB*- positive CC398/t034 strain from another pig herd, and one human MRSA strain and its isogenic Δ*agr* knockout mutant were used. The *agr*-negative strains adhered significantly better to human and porcine host cells than the *agr*-positive control strains. For the *agr*-positive porcine MRSA strains, cytotoxic effects on porcine mucosal cells were observed. The strong adhesive capacity of the naturally *agr*-negative livestock-associated MRSA in combination with diminished cytotoxic effects is likely favorable for inducing persistent colonization in pigs. Independent of the host cell type, similar adhesive capacities of the naturally *agr*-negative livestock-associated MRSA and the human MRSA strain were shown. Our results indicate that loss of *agr* in the livestock-

46 associated MRSA strain investigated in this study may have increased its potential to be
47 transmitted to and amongst humans.

# Introduction

Livestock-associated MRSA (LA-MRSA) strains have emerged worldwide. The strains typically belong to CC398 and are detected in particularly high rates among pigs [1-4]. LA-MRSA CC398 are thought to have originated from human methicillin-susceptible *Staphylococcus aureus* and acquired methicillin resistance during the host jump, at the same time losing genes important for colonization and infection in humans [5]. Even though LA-MRSA of CC398 lack important virulence factors, they have been suggested to exhibit an enhanced ability to acquire virulence factors through mobile genetic elements [6].

Transmission of CC398 from animals to humans has been described and occupational exposure to pigs was shown to increase nasal carriage rates (pig farmers: 20-86%; veterinarians: 4.6-45%) compared to the general population (0.8-3%) [2, 4, 7-14]. In addition, in recent years, there have been an increasing number of reports of MRSA CC398 strains causing invasive infections in humans without contact to livestock [15-18]. Therefore, the transmissibility and the virulence potential of LA-MRSA of CC398 need to be closely monitored.

Heikinheimo et al. [19] recently identified a new dominant *spa* type among LA-MRSA strains colonizing Finnish fattening pigs (CC398/t2741). Interestingly, for one CC398/t2741 strain isolated in this study, no genes within the accessory gene regulator locus (*agr*) or the fibronectin-binding protein B (*fnbB*) were detected by DNA microarray. The quorum sensing *agr* system is a major virulence gene regulator. Activation of *agr* has been shown to inhibit expression of certain cell-wall associated proteins and to increase exoprotein expression during the post-exponential phase of growth [20, 21]. While *agr* plays a major role in acute

infections, *agr*-defective mutants can frequently be isolated from bacteraemic patients and were linked to persistent infections [22] and increased mortality [23]. The *fnbB* gene encodes the fibronectin binding protein B (FnBPB), which belongs to the group of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). It facilitates adhesion of *S. aureus* to the components of the host cells and has a role in invasion [24]. Combined FnBPA and FnBPB function has been shown to be essential to induce severe infection [25]. The aim of this study was to characterize the naturally *agr/fnbB*-negative CC398/t2741 LA-MRSA strain detected in Finland with regard to growth, hemolysis, and adhesive capacity, and to describe the genomic background of the strain.

# Methods

## Bacterial strains

An overview of the strains used in this study is presented in Table 1. The naturally *agr/fnbB*-negative LA-MRSA strain investigated in this study (Fin47_H17) was isolated from the carcass of a fattening pig in Finland [19]. Two porcine LA-MRSA strains isolated in the same study were used as controls: Fin46_H17, an *agr*-positive, *fnbB*-negative isolate from the same herd (herd 17), and Fin48_H18, an *agr/fnbB*-positive isolate from another herd (herd 18). As additional controls, a MRSA strain that had caused recurring skin infections in humans (MN10) [26] and its isogenic *agr* knockout mutant (MN10_ Δ*agr*) were used. MN10_Δ*agr* was obtained by transduction of the *agr* knockout from RN6911 to MN10 using phage 80α and protocols previously described [27]. Correct deletion of *agr* in putative mutants grown on tetracycline selective plates was confirmed by PCR.

93

94 **Table 1: Strains used in this study.** Presence/absence of *agr* and *fnbB* is indicated as

95 previously determined by DNA microarray [19].

| Strain ID | Characteristics | Country | Year | Source [reference] |
|---|---|---|---|---|
| **Fin47_H17** | CC398/t2741, *agr*$^-$, *fnbB*$^-$ | FI | 2015 | Pig carcass, herd 17 [19] |
| **Fin46_H17** | CC398/t2741, *agr*$^+$, *fnbB*$^-$ | FI | 2015 | Pig nares, herd 17 [19] |
| **Fin48_H18** | CC398/t034, *agr*$^+$, *fnbB*$^+$ | FI | 2015 | Pig nares, herd 18 [19] |
| **MN10** | CC5/t002, *agr*$^+$, *fnbB*$^+$ | CH | 2013 | Human skin infection [26] |
| **MN10_Δ*agr*** | CC5/t002, NM10 *agr::tetM*, *agr*$^-$, *fnbB*$^+$ | CH | 2013 | This study |
| **RN27** | 80alpha lysogen | - | - | Brigitte Berger-Bächi [28] |
| **RN6911** | *agr* operon deleted (*agr::tetM*) | - | - | Brigitte Berger-Bächi [28] |

96

## Growth parameters

Single colonies were transferred from 5% sheep blood agar to 50 mL of Luria Bertani (LB) broth (Becton Dickinson, Allschwil, Switzerland) and grown for 24 h at 37°C (225 rpm). Growth of all strains in LB was determined by viable cell counts after 3 h, 6 h, 9 h, and 24 h using 10-fold dilution series and plate count agar (Sigma-Aldrich, Stockholm, Sweden), with incubation at 37°C for 18-24 h. Growth parameters such as exponential phase growth rate and maximum cell density were determined using DMFit 3.0 [29].

## Hemolysis

Screening for hemolytic activity was performed as previously described [30]. Briefly, alpha- and delta-hemolytic activity was determined by perpendicular streaking to the beta-hemolysin producing *S. aureus* reference strain RN4220 on 5% sheep blood agar and incubation over night at 37°C. In this assay, beta hemolysis results in a turbid zone. Alpha hemolytic activity of the test strain is inhibited by beta hemolysis of RN4220 where the strains intersect. Delta hemolysis of the test strain is synergistic with beta hemolysin of RN4220, resulting in an amplified zone of clearing where the strains intersect [31]. Gamma hemolytic activity cannot be detected in this assay, as it is inhibited by agar [32]. Subsequent cold shock (exposure to 4°C for 12h) was used to determine beta-hemolysis activity.

## Adhesion assay

Two different cell lines were used for adhesion assays: human cell line HaCaT (Human adult low Calcium high Temperature keratinocytes, CLS Cell Lines Service GmbH,

Eppelheim, Germany; [33]) and porcine cell line PT-K75 (porcine nasal turbinate/mucosa, CRL-2528, ATCC, Manassas, USA). The medium for the adhesion assay consisted of DMEM supplemented with 4.5 g/L glucose, 584 mg/L L-glutamine and 10% FCS (CLS Cell Lines Service GmbH) for the HaCaT cells and DMEM supplemented with 4.5 g/L glucose, 580mg/L L-glutamine, 110mg/L sodium pyruvate (GIBCO, Thermo Fisher Scientific, Invitrogen, Carlsbad, CA, USA) and 10% FCS (BioConcept, Allschwil, Switzerland) for the PT-K75 cells.

After at least one passage in a 75cm$^2$ canted neck tissue culture flask with vented cap (Corning, Sigma-Aldrich), HaCaT-cells were seeded at a concentration of $2 \times 10^5$ cells/well and PT-K75-cells at a concentration of $1.25 \times 10^5$ cells/well in a 24-well flat-bottom cell culture plate with low-evaporation lid (Techno Plastic Products AG, TPP, Trasadingen, Switzerland). The culture plates were incubated for 24 hours at 37°C and 5% $CO_2$ to reach a confluency of at least 80%.

Bacterial strains were prepared by growth on 5% sheep blood agar overnight at 37°C. Overnight cultures were prepared by inoculation of 5 mL LB broth with a single colony and incubation at 37°C (225 rpm shaking) for 15 hours. Cultures were subsequently adjusted to $OD_{590}$ = 0.40 (~$10^8$ CFU/ml) for each strain. The bacteria were used at a multiplicity of infection (MOI) of 10 to infect both cell lines (HaCaT and PT-K75) in 24-well plates. Uninfected cells were used as negative controls. After 15 min of incubation at 37°C, cells were imaged, before monolayers were washed eight times with Dulbecco's phosphate buffered saline (DPBS, GIBCO) and cells were harvested after five minutes of incubation with Trypsin-EDTA (0.25%, GIBCO) by scraping with a cell scraper (TPP). Bacterial cell counts were determined by 10-fold dilution series and plate count agar. Three independent

biological experiments were performed, each using three replicates per strain. For imaging purposes, the Nikon Eclipse Ti-U inverted microscope was used at 200-fold magnification with software NIS-Elements AR Analysis 4.3 (Nikon AG, Egg, Switzerland).

## Statistical analysis

All statistical analyses with the exception of growth parameter analyses were performed using R, 3.2. Results were compared using a gaussian linear model of the form count~strain+host.cellline. Growth parameters were compared between strains using SPSS Statistics 22 (SPSS Inc.Chicago, Illinois). Growth parameters such as exponential phase growth rate and maximum cell density were determined using DMFit 3.0 (Baranyi & Roberts, 1994) and compared using one-way ANOVA. Differences were considered statistically significant if $p < 0.05$.

## Whole genome sequencing

DNA was extracted from overnight cultures grown on 5% sheep blood agar plates using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The porcine LA-MRSA strains Fin47_H17, Fin46_H17, and Fin48_H18 were sequenced on the Illumina Miseq platform with 250bp paired end reads following NEBNextUltra library creation. Raw sequencing reads were adaptor and quality trimmed and filtered using Trimmomatic version 0.32 [34]. Mapping against the CC398 reference strain S0385 (EMBL Accession AM990992) [6] using BWA [35] indicated chromosome coverage of each genome to be: Fin47_H17 = 132.8x ± 76.2, Fin46_H17 =

140.6 x ±71.2, Fin48_H18 = 115.1x ± 53.7. *De novo* assembly used SPAdes v 3.1.0 in multi-cell mode [36]. Scaffolds were rearranged in ACT [37] against S0385 using files generated in WebACT (http://www.webact.org/). Resulting assemblies comprise 28, 25 and 27 scaffolds respectively, with scaffolds under 1kb, which did not match the reference, excluded. Target coding sequences (CDSs) involved in adhesion were identified and analysed in all genomes using ACT and, where necessary, single CDSs were aligned using ClustalW. The phylogeny was generated in http://wgsastaging.pathogensurveillance.net/ using available assembled CC398 genomes for context [6, 38-42]. Antibiotic resistance phenotypes were also predicted using this online tool.

All read data and assemblies have been deposited with ENA under project number (PRJEB14187).

# Results

## Growth parameters

No statistically significant differences were observed between growth of the five strains in LB broth with regard to exponential phase growth rate or maximum cell density (Fig. 1). However, the naturally *agr*-negative LA-MRSA Fin47_H17 exhibited the lowest exponential phase growth rate ($\mu_{max}$ = 0.50) compared to all other strains ($\mu_{max}$ = 0.51-0.57). Fin47_H17 also reached the lowest maximum cell density (8.97 log CfU/mL) compared to all other strains tested (9.70-9.91 log CfU/mL).

**Fig. 1: Growth of all tested strains in LB over 24h.** The tested strains exhibited no statistically significant differences in exponential phase growth rate or maximum cell density reached (error bars: 95% CI).

## Haemolysis pattern

Hemolysis patterns after incubation at 37°C overnight and after subsequent cold exposure are shown in Fig. 2. No hemolysis was visible for both the naturally *agr*-negative Fin47_H17 strain and *agr* knockout mutant MN10_ Δ*agr*. Parental strain MN10 exhibited alpha and delta hemolysis. Fin46_H17 exhibited alpha and beta hemolysis, and Fin48_H18 exhibited beta and delta hemolysis.

**Fig. 2: Hemolysis.** The *agr*-negative strains exhibited no hemolysis (Fin47_H17 and MN_Δ*agr*). MN10 exhibited alpha and delta hemolysis, Fin46_H17 exhibited alpha and beta hemolysis, and Fin48_H18 exhibited beta and delta hemolysis.

## Adhesion properties

While no morphological changes were observed in HaCaT cells after bacterial infection, infection of PT-K75 cells with the *agr*-positive porcine LA-MRSA strains (Fin46_H17 and Fin48_H18) resulted in a cytotoxic effect including shrinking, rounding and detachment of the cells (Fig. 3). To minimize cytotoxicity, a trial experiment was performed on PT-K75 cells with an MOI of 1: cytotoxic effects were still observed (data not shown). In contrast, neither the naturally *agr*-negative porcine LA-MRSA strain Fin47_H17 nor the human-associated MRSA strain MN10 and its isogenic *agr* knockout mutant induced any morphological changes in the PT-K75 cells (Fig. 3).

Counts of adherent bacteria revealed that the *agr*-negative strains Fin47_H17 and MN10_Δ*agr* displayed increased adhesive capacity in both human HaCaT and porcine PT-K75 cells (Fig. 4) compared to the *agr*-positive LA-MRSA and the human-associated parental MRSA strain MN10, respectively. Median colony counts for Fin47_H17 were 3,600 for HaCaT and 14,000 for PT-K75 cells per culture well. These results show significantly higher adhesive capacity of this strain compared to the *agr*-positive strains Fin46_H17 (790 to HaCaT, p = 1.3e-06), Fin48_H18 (500 to HaCaT, p = 2.0e-07) or MN10 (1,200 to HaCaT, $p$ = 4.3e-13; 7,200 to PT-K75, $p$ = 0.001). The median colony counts for the MN10_Δ*agr* mutant strain (3,190 to HaCaT and 35,000 to PT-K75) were significantly higher than those of MN10 (1,200 to HaCaT, $p$ = 0.003; 7,200 to PT-K75, $p$ = 4.5e-05). Thus, the naturally *agr*-

219 negative LA-MRSA and MN10_Δ*agr* exhibited significantly higher adhesive capacity than
220 the *agr*-positive LA-MRSA strains and parental strain MN10, respectively. Overall, the tested
221 porcine and human MRSA strains adhered significantly better to the porcine than to the
222 human cells ($p$ = 1.3e-06).
223
224 **Fig. 3: Cytotoxic effects on porcine cells.** Representative images of the two cell lines before
225 ($T_0$) and shortly after ($T_{15\ min}$) infection. Bacterial strains used were the porcine LA-MRSA
226 strains Fin47_H17, Fin46_H17, and Fin48_H18, as well as the MRSA strain linked to human
227 infections (MN10) and its isogenic knockout mutant MN10_Δ*agr*. Cytotoxic effects of the
228 *agr*-positive porcine LA-MRSA strains Fin46_H17 and Fin48_H18 on PT-K75 cells can be
229 identified at $T_{15\ min}$.
230
231 **Fig. 4: Adhesion to A) human cells and B) porcine cells: colony counts for different**
232 **strains.** The plots indicate the median (horizontal line), the range from the first to the third
233 quartile (25–75%; box), and the extreme values (whiskers) of the colony counts for each
234 strain after adhesion to A) human cells (HaCaT) and B) porcine cells (PT-K75). The data
235 resulted from three independent biological experiments with three replicates for each strain
236 (n=9).

## Genomic analysis

Whole genome sequencing and phylogenetic analysis of strains within CC398 confirmed that Fin47_H17 is closely related to Fin46_H17, with Fin48_H18 more distantly related (S1 Fig).

In the naturally *agr*-negative LA-MRSA Fin47_H17, a deletion of 3,789 bp covering *hlD*, *agrB* and *agrC* (Fig. 5A) was identified. In both this strain and the closely related strain Fin46_H17, a further deletion completely removing *fnbB* was detected that leaves the adjacent *fnbA* intact (Fig. 5B). However, as *fnbB* in both Fin48_H18 and reference strain S0385 is truncated after 349 amino acids with a single nucleotide mutation causing a premature stop codon, the functional relevance of this is unclear.

Genes associated with adhesion were investigated within the genomes (S2 Table). Such genes often have varying repeat lengths, and this was identified in these strains in the genes: *spa*, *sdrC* and the hypothetical protein encoding CDS *SAPIG1791*; with the further genes *coa*, *sdrD*, *clfA*, *clfB, ebhA* and *cna* almost identical between the three porcine LA-MRSA strains, but variable in comparison to homologues in S0385. The *vwb* gene, encoding a secreted von Willebrand factor-binding protein, was found to be absent at the equivalent chromosomal locus in Fin48_H18, with a 13.7 kb deletion covering this region. All other genes investigated were found to be identical or almost identical to those in S0385 (*eno*, *isdB*, *isdA*, *ecb*, *fib*, *ebpS*, *map*, *sdrH*, *agrA*, *isaB*).

The strains carry alternative complements of plasmids and phage (S2 Table) and the predicted antibiotic resistance profiles (Fin47_H17 and Fin46_H17: $PEN^R$, $TET^R$, $ERY^R$,

259 $MET^R$; Fin 48_H18: $PEN^R$, $TET^R$, $MET^R$, $TRI^R$) largely correspond to those phenotypically
260 determined previously, with the exception of clindamycin [19].
261
262 **Fig. 5: Comparison of genome loci around *agr* and *fnbB*.** Each line refers to a genomic
263 section in the strain indicated. Arrows indicate CDSs. Grey bars show homology between
264 loci according to the scale. **A.** *agr* locus showing the extent of the deletion in Fin47_H18,
265 covering *SAPIG2071-2075*. *hlD* is shown in yellow and the *agr* locus (*agrBCA*) in green.
266 Only *argA* remains in Fin47_H17. **B.** *fnbB* locus showing the deletion of *fnbB* (*SAPIG2551*)
267 in both Fin47_H17 and Fin46_H17. *fnbB* in both S0385 and Fin48_H18 is truncated by a
268 stop codon and is shown in brown for a pseudogene. *fnbA* (yellow) displays some homology
269 to *fnbB*. Figure was drawn in Easyfig [43].
270

271 # Discussion

272 Heikinheimo et al. [19] investigated LA-MRSA isolated from Finnish fattening pigs
273 at slaughter. They found that CC398/t2741 strains were predominant among the isolated LA-
274 MRSA. Interestingly, one of the isolated strains (Fin47_H17) did not harbor genes from the
275 *agr* locus. In this study, we determined the genomic basis of this loss of *agr* and its effect on
276 growth, hemolytic activity, and adhesive capacity to human and porcine cell lines.
277 Loss of *agr* did not result in altered growth parameters, but had a strong impact on
278 hemolytic activity consistent with previous studies suggesting that expression of alpha and
279 delta hemolysins is strongly induced by *agr* [30, 31]. Hemolysins are widely recognized as

280 important virulence factors in *S. aureus* infections. However, selective survival of *agr*-
281 defective non-hemolytic *S. aureus* in wound and abscess models has been demonstrated [44].
282 In this study, both *agr*-negative strains tested exhibited increased adhesive capacity to
283 both human and porcine cells. These results are consistent with previous findings suggesting
284 *agr* to inhibit the expression of adhesion proteins [45-48]. Loss of *agr* was shown to
285 significantly increase adhesive capacity to human endothelial [49] and mesothelial cells [50].
286 In a bovine mammary epithelial cell line (MAC-T), two- to threefold higher numbers of
287 internalized viable bacteria of an *agr* mutant were recovered compared to the wild type [51].
288 LA-MRSA CC398 was reported to cause more cell damage within epithelial cells
289 than community-associated and hospital-acquired MRSA [52]. In this study, pronounced
290 cytotoxic effects of the *agr*-positive porcine LA-MRSA strains on porcine cells were
291 identified. In contrast, the closely related *agr*-negative porcine LA-MRSA Fin47_H17 did not
292 induce cytotoxic effects. This may be due to reduced expression levels of *agr*-dependent
293 cytotoxic proteins such as alpha hemolysin [53]. It is also consistent with a previous study
294 reporting cytotoxic effects in MAC-T cells upon infection with RN6390, but no cytotoxic
295 effects in *agr* mutant strain RN6911 [51]. The finding that porcine *agr*-positive LA-MRSA
296 only led to cytotoxicity in the porcine cell line could be due to host-specific factors
297 influencing susceptibility to alpha hemolysin [54].
298 Whole genome sequencing revealed that the *fnbB* gene in Fin48_H18 is truncated, potentially
299 impairing functionality of FnBPB, whereas it is fully deleted from the genomes of strains
300 Fin47_17 and Fin46_17. We observed no significant differences concerning adherence to
301 porcine cells of Fin48_H18 and Fin46_17. For strain Newman, it has been suggested that
302 truncation of *fnbB* was transferred to *fnbA,* resulting in complete secretion FnBPs to the

culture medium and loss of cell wall anchor function [55]. Consistent with these findings, reduced adhesive capacity of a *fnbAB* mutant to HaCaT cells has been described [56].

We observed no significant differences in adhesive capacity between porcine and human MRSA apart from the described influence of *agr*. However, irrespective of strain origin (LA-MRSA or human MRSA), adherence to porcine cells significantly exceeded adherence to human cells. LA-MRSA CC398 do not only colonize livestock, but can also be transmitted to humans with direct livestock exposure or even between humans. Although decreased adherence of LA-MRSA compared to community/hospital-associated MRSA to human endothelial and epithelial cells was observed [52], LA-MRSA strains may rapidly adapt to the human host. Our results suggest that LA-MRSA are capable of adherence to both porcine and human cells with the same efficiency as a community-acquired MRSA control strain linked to recurring infections within a family [26].

# Conclusion

We were able to show that the recently reported naturally *agr*-negative and *fnbB*-negative LA-MRSA strain Fin47_H17 exhibits high adhesive capacity to porcine and human cells. Our results suggest that loss of *agr* leads to increased adhesive capacity and that porcine LA-MRSA can exhibit pronounced *agr*-dependent cytotoxic effects on porcine cells. Our findings provide further evidence for the importance of LA-MRSA as an emerging public health concern.

# Acknowledgments

The authors are grateful to Prof. Roger Stephan, Prof. Angelika Lehner, Dr. Taurai Tasara, Dr. Claudia Guldimann and Dr. Henna-Maria Sihto from the Institute for Food Safety and Hygiene, University of Zurich and Dr. Cory Ann Leonard, Dr. Hanna Marti, med. vet. Sabrina Wanninger and med. vet. Jasmin Kuratli from the Institute for Veterinary Pathology, University of Zurich, Vetsuisse Faculty, for helpful advice, discussion and laboratory support. We also thank the Functional Genomics Centre Zurich for technical assistance.

# Conflict of interest statement

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publication of this article.

# References

Alt K, Fetsch A, Schroeter A, Guerra B, Hammerl JA, Hertwig S, et al. Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. BMC Vet Res. 2011;7:69. doi: 10.1186/1746-6148-7-69.

Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, Moritz ED, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One. 2009;4(1):e4258. doi: 10.1371/journal.pone.0004258.

345

346 de Neeling AJ, van den Broek MJ, Spalburg EC, van Santen-Verheuvel MG, Dam-Deisz
347 WD, Boshuizen HC, et al. High prevalence of methicillin resistant *Staphylococcus aureus* in
348 pigs. Vet Microbiol. 2007;122(3-4):366-72

349

350 Khanna T, Friendship R, Dewey C, Weese JS. Methicillin resistant *Staphylococcus aureus*
351 colonization in pigs and pig farmers. Vet Microbiol. 2008;128(3-4):298-303.

352

353 Price LB1, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, et al. *Staphylococcus*
354 *aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. MBio.
355 2012;3(1). pii: e00305-11. doi: 10.1128/mBio.00305-11. Print 2012.

356

357 Schijffelen MJ, Boel CH, van Strijp JA, Fluit AC 2010. Whole genome analysis of a
358 livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of
359 human endocarditis. BMC Genomics 2010;11: 376. doi: 10.1186/1471-2164-11-376

360

361 Ellis MW, Hospenthal DR, Dooley DP, Gray PJ, Murray CK. Natural history of community-
362 acquired methicillin-resistant *Staphylococcus aureus* colonization and infection in soldiers.
363 Clin Infect Dis. 2004;39(7):971-9.
364 Kuehnert MJ, Kruszon-Moran D, Hill HA, McQuillan G, McAllister SK, Fosheim G, et al.
365 Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. J
366 Infect Dis. 2006;193(2):172-9.

367

368 Wulf M, van Nes A, Eikelenboom-Boskamp A, de Vries J, Melchers W, Klaassen C, et al.
369 Methicillin-resistant *Staphylococcus aureus* in veterinary doctors and students, the
370 Netherlands. Emerg Infect Dis. 2006;12(12):1939-41.
371
372 Wulf MW, Sørum M, van Nes A, Skov R, Melchers WJ, Klaassen CH, et al. Prevalence of
373 methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study. Clin
374 Microbiol Infect. 2008;14(1):29-34.
375
376 van Belkum A, Melles DC, Peeters JK, van Leeuwen WB, van Duijkeren E, Huijsdens XW,
377 et al. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs
378 and humans. Emerg Infect Dis. 2008;14(3):479-83. doi: 10.3201/eid1403.070760.
379
380 Cuny C, Nathaus R, Layer F, Strommenger B, Altmann D, Witte W. Nasal colonization of
381 humans with methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 with and without
382 exposure to pigs. PLoS One. 2009;4(8):e6800. doi: 10.1371
383
384 Denis O, Suetens C, Hallin M, Catry B, Ramboer I, Dispas M, et al. Methicillin-resistant
385 *Staphylococcus aureus* ST398 in swine farm personnel, Belgium. Emerg Infect Dis.
386 2009;15(7):1098-101. doi: 10.3201/eid1507.080652.
387
388 Huber H, Giezendanner N, Stephan R, Zweifel C. Genotypes, Antibiotic Resistance Profiles
389 and Microarray-Based Characterization of Methicillin-Resistant *Staphylococcus aureus*
390 Strains Isolated from Livestock and Veterinarians in Switzerland. Zoonoses Public Health.
391 2011;58(5):343-9. doi: 10.1111/j.1863-2378.2010.01353.x.

Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert

392

393 Wulf MW, Markestein A, van der Linden FT, Voss A, Klaassen C, Verduin CM. First
394 outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June
395 2007. Euro Surveill. 2008;13(9). pii: 8051.

396

397 Valentin-Domelier AS, Girard M, Bertrand X, Violette J, François P, Donnio P-Y, et al.
398 Methicillin-susceptible ST398 *Staphylococcus aureus* responsible for bloodstream infections:
399 an emerging human-adapted subclone? PLoS One. 2011;6:e28369.
400 10.1371/journal.pone.0028369

401

402 Uhlemann AC, Porcella SF, Trivedi S, Sullivan SB, Hafer C, Kennedy AD, et al.
403 Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398
404 clone with distinct genomic and cell adhesion properties. MBio. 2012;3(2). pii: e00027-12.
405 doi: 10.1128/mBio.00027-12.

406

407 Verkade E, Bergmans AM, Budding AE, van Belkum A, Savelkoul P, Buiting AG, et al.
408 Recent emergence of *Staphylococcus aureus* clonal complex 398 in human blood culture.
409 PLoS ONE 2012;7:e41855.

410

411 Heikinheimo A, Johler S, Karvonen L, Julmi J, Fredriksson-Ahomaa M, Stephan R. New
412 dominant spa type t2741 in livestock-associated MRSA (CC398-MRSA-V) in Finnish
413 fattening pigs at slaughter. Antimicrob Resist Infect Control. 2016;5:6. doi: 10.1186/s13756-
414 016-0105-8.

415

Bronner S, Monteil H, Prevost G. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol. Rev. 2004;28:183–200.

Tseng CW, Stewart GC. Rot repression of enterotoxin b expression in *Staphylococcus aureus*. J. bacteriol., 2005 July 21. ed. 187:5301–5309.

Fowler VGJr, Sakoulas G, McIntyre LM, Meka VG, Arbeit RD, Cabell CH, et al. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. J Infect Dis. 2004;190:1140–9.

Schweizer ML, Furuno JP, Sakoulas G, Johnson JK, Harris AD, Shardell MD, et al. Increased mortality with accessory gene regulator (agr) dysfunction in *Staphylococcus aureus* among bacteremic patients. Antimicrob Agents Chemother. 2011;55:1082–7.

Foster TJ, Geoghegan JA, Ganesh VK, Höök M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 2013;12:49–62.

Shinji H, Yosizawa Y, Tajima A, Iwase T, Sugimoto S, Seki K, et al. Role of fibronectin-binding proteins A and B in in vitro cellular infections and in vivo septic infections by *Staphylococcus aureus*. Infect Immun. 2011;79:2215–23.

439 Nüesch-Inderbinen MT, Stalder U, Johler S, Hächler H, Stephan R, Nüesch H-J. Intrafamilial
440 spread of a Panton-Valentine leukocidin-positive community-acquired methicillin-resistant
441 *Staphylococcus aureus* belonging to the paediatric clone ST5 SSCmecIV. JMM Case Reports
442 2014 Sept 12. doi:10.1099/jmmcr.0.001859.
443
444 Charpentier E, Anton AI, Barry P, Alfonso B, Fang Y, Novick RP. Novel cassette-based
445 shuttle vector system for gram-positive bacteria. Appl Environ Microb. 2004;70:6076–85.
446
447 Novick RP. Analysis by transduction of mutations affecting penicillinase formation in
448 *Staphylococcus aureus*. J Gen Microbiol. 1963;33:121–36.
449
450 Baranyi J, Roberts TA. A dynamic approach to predicting bacterial growth in food. Int J Food
451 Microbiol. 1994;23(3-4):277-94.
452
453 Herbert S, Ziebandt AK, Ohlsen K, Schäfer T, Hecker M, Albrecht D, et al. Repair of global
454 regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical
455 isolates. Infect Immun. 2010;78(6):2877-89. doi: 10.1128/IAI.00088-10.
456
457 Adhikari RP, Arvidson S, Novick RP. A nonsense mutation in agrA accounts for the defect in
458 agr expression and the avirulence of *Staphylococcus aureus* 8325-4 traP::kan. Infect. Immun.
459 2007;75:4534–4540.
460
461 Wiseman, GM. The hemolysins of *Staphylococcus aureus*. Bacteriol. Rev. 1975;39:317–344.
462

Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert

463 Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal
464 keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell
465 Biol. 1988;106(3):761-71.
466
467 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
468 data. Bioinformatics 2014;30:2114-2120. doi: 10.1093/bioinformatics/btu170
469
470 Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler Transform.
471 Bioinformatics 2009;25:1754-1760.
472
473 Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a
474 new genome assembly algorithm and its applications to single-cell sequencing. J Comput
475 Biol 2012;19: 455-477. doi: 10.1089/cmb.2012.0021.
476
477 Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J. ACT: the
478 Artemis Comparison Tool. Bioinformatics 2005;21:3422-3423. doi:
479 10.1093/bioinformatics/bti553
480
481 Uhlemann AC, Porcella SF, Trivedi S, Sullivan SB, Hafer C, Kennedy AD, et al.
482 Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398
483 clone with distinct genomic and cell adhesion properties. MBio. 2012 Feb 28;3(2). pii:
484 e00027-12. doi: 10.1128/mBio.00027-12. Print 2012.
485

van der Mee-Marquet N, Hernandez D, Bertrand X, Quentin R, Corvaglia AR, François P. Whole-Genome Sequence of the Ancestral Animal-Borne ST398 *Staphylococcus aureus* Strain S123. Genome Announc. 2013 Aug 29;1(5). pii: e00692-13. doi: 10.1128/genomeA.00692-13.

Hernandez D, van der Mee-Marquet N, Kluytmans J, Donnio PY, Quentin R, Corvaglia AR, et al. Whole-Genome Sequences of *Staphylococcus aureus* ST398 Strains of Animal Origin. Genome Announc. 2013 Sep 26;1(5). pii: e00689-13. doi: 10.1128/genomeA.00689-13.

Corvaglia AR1, François P, Bertrand X, Quentin R, Hernandez D, van der Mee-Marquet N. Whole-Genome Sequences of Two *Staphylococcus aureus* ST398 Strains of Human Origin, S94 and S100. Genome Announc. 2013 Aug 29;1(5). pii: e00691-13. doi: 10.1128/genomeA.00691-13.

Golding GR, Bryden L, Levett PN, McDonald RR, Wong A, Graham MR, et al. Whole-genome sequence of livestock-associated st398 methicillin-resistant *staphylococcus aureus* Isolated from Humans in Canada. J Bacteriol. 2012 Dec;194(23):6627-8. doi: 10.1128/JB.01680-12.

Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011 Apr 1;27(7):1009-10. doi: 10.1093/bioinformatics/btr039.

508 Schwan WR, Langhorne MH, Ritchie HD, Stover CK. Loss of hemolysin expression in
509 *Staphylococcus aureus* agr mutants correlates with selective survival during mixed infections
510 in murina abscesses and wounds. FEMS Immunol Med Microbiol. 2003;38(1):23-8.
511
512 Patel AH, Kornblum J, Kreiswirth B, Novick R, Foster TJ. Regulation of the protein A-
513 encoding gene in *Staphylococcus aureus*. Gene. 1992;114:25–34
514
515 Saravia-Otten P, Müller HP, Arvidson S. Transcription of *Staphylococcus aureus* fibronectin
516 binding protein genes is negatively regulated by agr and an agr-independent mechanism. J.
517 Bacteriol. 1997;179:5259–5263.
518
519 Projan SJ, Novick RP. The molecular basis of pathogenicity. In: Crossley KB, Archer GL,
520 editors. The staphylococci in human disease. Churchill Livingstone, New York, N.Y; 1997.
521 pp 55–82.
522
523 Wolz C, Pöhlmann-Dietze P, Steinhuber A, Chien YT, Manna A, van Wamel W, et al. Agr-
524 independent regulation of fibronectin-binding protein(s) by the regulatory locus sar in
525 *Staphylococcus aureus.* Mol Microbiol. 2000;36(1): 230-243.
526
527 Pöhlmann-Dietze P, Ulrich M, Kiser KB, Döring G, Lee JC, Fournier JM, et al. Adherence of
528 *Staphylococcus aureus* to endothelial cells: influence of capsular polysaccharide, global
529 regulator agr, and bacterial growth phase. Infect Immun. 2000;68(9):4865-71.
530

Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert
Feldfunktion geändert

Poston SM, Glancey GR, Wyatt JE, Hogan T, Foster TJ. Co-elimination of mec and spa genes in *Staphylococcus aureus* and the effect of agr and protein A production on bacterial adherence to cell monolayers. J Med Microbiol. 1993;39(6):422-8.

Wesson CA, Liou LE, Todd KM, Bohach GA, Trumble WR, Bayles KW. *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis. Infect Immun. 1998;66(11):5238-43.

Ballhausen B, Jung P, Kriegeskorte A, Makgotlho PE, Ruffing U, von Müller L, et al. LA-MRSA CC398 differ from classical community acquired-MRSA and hospital acquired-MRSA lineages: functional analysis of infection and colonization processes. Int J Med Microbiol. 2014;304(7):777-86. doi: 10.1016/j.ijmm.2014.06.006.

Ezepchuk YV, Leung DY, Middleton MH, Bina P, Reiser R, Norris DA. Staphylococcal toxins and protein A differentially induce cytotoxicity and release of tumor necrosis factor-alpha from human keratinocytes. J Invest Dermatol. 1996;107(4):603-9.

Popov LM, Marceau CD, Starkl PM, Lumb JH, Shah J, Guerrera D, et al. The adherens junctions control susceptibility to *Staphylococcus aureus* α-toxin. Proc Natl Acad Sci U S A. 2015;112(46):14337-42. doi: 10.1073/pnas.1510265112.

Grundmeier M, Hussain M, Becker P, Heilmann C, Peters G, Sinha B. Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient

Feldfunktion geändert

adherence and host cell invasion due to loss of the cell wall anchor function. Infect Immun. 2004;72(12):7155-63.

Bur S, Preissner KT, Herrmann M, Bischoff M. The *Staphylococcus aureus* extracellular adherence protein promotes bacterial internalization by keratinocytes independent of fibronectin-binding proteins. J Invest Dermatol. 2013;133(8):2004-12. doi: 0.1038/jid.2013.87.

Feldfunktion geändert

Feldfunktion geändert

Feldfunktion geändert

Feldfunktion geändert

# Supporting information

**S1 Fig. Phylogenetic tree.** Phylogenetic tree of whole genome sequenced isolates in the context of other sequenced CC398 strains. Scale bar shows the number of substitutions within the expected core genome (1,799,838 bp).

**S2 Table. Variable elements.** Table providing an overview of variable elements within the genomes of porcine LA-MRSA strains Fin47_H17, Fin46_17, and Fin48_H18 compared to reference strain S0385.

576

577

578

579

580

581

582 **Supplementary Table 1: Genomic analysis of adhesion associated genes.** S0385 data from Uhlemann 2012, with additions. Abbreviations:
583 V/S = variable/same (compared to strain indicated in superscript), A = absent at this location, I = insertion, X = aa substitution, ψ = pseudogenes,
584 wt = wild type, aa =amino acid, Δ = deletion, SNP = single nucleotide polymorphism.

| **Gene** | **S0385 locus tag[1] SAPIG** | **S085** | **NM01** | **Fin47_H17** | **Fin46_H17** | **Fin48_H18** | **Comment** |
|---|---|---|---|---|---|---|---|
| *spa* | 0122 | 8 aa Δ at aa390 | 2 X at aa390 | wt | wt | I (8 aa at aa390) | Variable repeat length |
| *coa* | 0240 | 81 bp Δ | wt | wt | wt | wt | Variable repeat length |
| *vwb* | 0483 | wt | A | $V^{S0385}$ | $V^{S0385}$ | A (at this location) | Variable sequence/presence |
| *sdrC* | 0636 | 174 bp Δ | wt | 1 SNP | $S^{Fin47\_H17}$ | I (24aa at aa737) | Variable repeat length |
| *sdrD* | 0637 | 42 bp Δ and 4 X | Δ 54 bp | wt | wt | wt | Variable repeat length |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | and SNPs |
| *eno* | 0855 | | | $S^{S0385}$ | $S^{S0385}$ | 1 SNP, non synonymous | |
| *clfA* | 0866-7 | ψ truncated, 2 frameshifts | wt | $V^{S0385}$ | $S^{Fin47\_H17}$ | $V^{S0385}$ | Variable repeat length |
| *isdB* | 1125 | SNP, 9 bp Δ | wt | $S^{S0385}$ | $S^{S0385}$ | $I^{S0385}$ | |
| *isdA* | 1126 | | | $S^{S0385}$ | $S^{S0385}$ | 1 SNP, synonymous | |
| *ecb* | 1150 | wt | wt | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| *fib* | 1154 | wt | wt | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| *ebhA* | 1434 | 53 aa Δ at aa9851 and 1 X | wt | 1 aa X | 1 X | 2 X | Variable repeat length |
| *ebpS* | 1480 | wt | wt | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| hypothetical | 1791 | 174 bp Δ | wt | 1 aa change | $S^{Fin47\_H17}$ | wt | Variable repeat length |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | (aa39) | | | |
| *map* | 1981 | | | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| *sdrH* | 2069 | wt | Δ 39 bp | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| *hlD* | 2072 | wt | wt | ψ A | wt | wt | Variably present |
| *agrB* | 2073 | wt | wt | A | wt | wt | Variably present |
| *agrA* | 2076 | | | $S^{S0385}$ | $S^{S0385}$ | $S^{S0385}$ | |
| *fnbB* | 2550-1 | ψ truncated, stop codon | wt | Deleted | Deleted | ψ truncated, stop codon | Often defunct |
| *fnbA* | 2553 | wt | wt | Δ9 bp in repeat region, 12 aa changes | $S^{Fin47\_H17}$ | wt | Variable |
| *clfB* | 2679 | ψ truncated | Δ42aa at aa819 | wt | wt | wt | Variable repeats / truncated |
| *isaB* | 2688 | | | $S^{S0385}$ | $S^{S0385}$ | 1 SNP, | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | synonymous | |
| *cna* | 2740 | wt | ΔB domain | Δ187aas, 4 X | As Fin47 | Δ187aas, 4 X | Variable in this clade |

585